Effects of intramammary infusion of cis–urocanic acid on mastitis-associated inflammation and tissue injury in dairy cows

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Objective—To evaluate the effects of cis–urocanic acid (cis-UCA) on mammary gland (MG) inflammation and injury associated with *Escherichia coli*–induced mastitis in dairy cows.

Animals—12 lactating dairy cows (36 MGs).

Procedures—At 7-week intervals, a different MG in each cow was experimentally inoculated with *E coli*. At 6-hour intervals from 6 to 36 hours after inoculation, the inoculated MG in each cow was infused with 40 mL of saline (0.9% NaCl) solution, 12.5mM cis-UCA, or 25mM cis-UCA (4 cows/group); ultimately, each cow received each treatment. Immediately prior to and at various time points after inoculation and treatment, milk samples were collected. Bacterial CFUs, somatic cell counts (SCCs), N-acetyl-beta-D-glucosaminidase (NAGase) and lactate dehydrogenase (LDH) activities, and concentrations of bovine serum albumin, tumor necrosis factor-α, and cis-UCA were quantified in each milk sample.

Results—Compared with findings in saline solution–treated MGs, NAGase and LDH activities in milk samples from cis-UCA–treated MGs were lower. Cis-UCA had no effect on milk SCCs and milk concentrations of bovine serum albumin and tumor necrosis factor-α. Furthermore, cis-UCA had no adverse effect on bacterial clearance; CFUs of *E coli* in MGs treated with saline solution or cis-UCA were equivalent.

Conclusions and Clinical Relevance—In cows, milk NAGase and LDH activities were both lower in *E coli*–infected MGs infused with cis-UCA than in those infused with saline solution, which suggests that cis-UCA reduced mastitis-associated tissue damage. Furthermore, these data indicated that therapeutic concentrations of cis-UCA in milk can be achieved via intramammary infusion. (*Am J Vet Res* 2009;70:373–382)

Mastitis is among the most prevalent diseases of dairy cows and is one of the most costly diseases in the animal agricultural sector. Annual economic losses associated with mastitis have been estimated to approach $2 billion in the United States and $35 billion worldwide.[1,2] These losses in revenue arise as a consequence of decreased milk production, lowered milk quality, veterinary medical and antimicrobial costs, indirect treatment costs as a result of milk withholding, and expenses incurred for animal replacement.[3]

Although the implementation of certain management practices has reduced the number of cases of mastitis caused by contagious pathogens, the incidence of mastitis caused by environmental pathogens, such as gram-negative bacteria, has remained constant or increased.[4–6] Among the gram-negative bacteria, *Escherichia coli* remains the most prevalent cause of clinical mastitis in dairy cows.[6–8] Intramammary infections caused by *E coli* are typically more severe and are associated with a higher mortality rate than those caused by other pathogens.[9–11] There are conflicting reports[12–15] on the efficacy of existing vaccines to reduce the severity of *E coli* infections, and the effectiveness of antimicrobials approved for the treatment of these infections remains suboptimal.[16]

In response to intramammary infection, neutrophils are rapidly recruited to the mammary gland.

Abbreviations

BSA	Bovine serum albumin
HPLC	High-performance liquid chromatography
LDH	Lactate dehydrogenase
NAGase	N-acetyl-beta-D-glucosaminidase
ROS	Reactive oxygen species
RPM	Revolutions per minute
SCC	Somatic cell count
TNF-α	Tumor necrosis factor-α
UCA	Urocanic acid

Received March 25, 2008.
Accepted June 23, 2008.
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Within several hours of mammary gland infection in cattle, the neutrophil concentration within milk can exceed 50 million cells/mL.[17] Once recruited to the site of infection, these cells produce a variety of molecules with bactericidal activity, including ROS.[18,19] Although generation of ROS is critical for host defense against infection, extracellular release of ROS can induce indiscriminate injury to host tissues.[20–22] Several in vitro studies[23–27] have revealed that activated neutrophils induce direct injury to bovine mammary gland epithelium and that scavengers of ROS protect against this injury. Results of in vivo studies[28–31] have indicated that the activities of several biomarkers of mammary gland tissue injury, including NAGase and LDH, are increased in milk during episodes of mastitis in cattle. Furthermore, intramammary administration of an antioxidant reduces bacterial endotoxin-induced mammary tissue injury in dairy cows.[32] Together, these data indicate a role for neutrophil-derived ROS in the pathogenesis of the mammary gland tissue injury that accompanies mastitis.

Cis-UCA, which is formed in response to UV radiation–induced photoisomerization of the trans isomer that is naturally found in skin, is a molecule that has anti-inflammatory and immunosuppressive properties.[33] Cis-UCA suppresses cell-mediated immunity associated with both adaptive[34–37] and innate immune responses.[38,39] The generation of ROS by neutrophils, which is known to contribute to the tissue injury associated with the influx of these cells into infected tissues, is decreased by cis-UCA.[40,41] Cis-UCA is able to exert this effect without impairing neutrophil-mediated killing of bacteria. This latter finding is ascribed to the ability of cis-UCA to selectively inhibit extracellular, but not intracellular, generation of ROS.[41]

Because mastitis is characterized by an influx of large numbers of neutrophils into mammary glands, cis-UCA may have therapeutic application for reducing the inflammation-mediated injury associated with this disease without compromising host innate immune responses that are essential for bacterial clearance. The objective of the study reported here was to evaluate the effect of intramammary infusion of cis-UCA on biomarkers of inflammation and tissue injury in dairy cows with experimentally induced acute clinical mastitis.

Materials and Methods

Animals—Twelve clinically normal, lactating Holstein cows were selected from the USDA Agricultural Research Service Beltsville Agricultural Research Center herd. Among these cows, the mean ± SE duration of lactation was 241 ± 28 days. The use and care of all animals in the study were approved by the USDA Agricultural Research Service Beltsville Agricultural Research Center's Animal Care and Use Committee.

Preparation of cis-UCA—Trans-UCA[a] was admixed in reverse-osmosis water alkalinized with solid NaOH. The mixture was irradiated with a broadband (250 to 350 nm) UV light source until HPLC analysis indicated that the photostationary state of the cis and trans isomers was reached. The mixture was evaporated to dryness, and the residue was dissolved into a small amount of water. The isomers were separated via anion exchange chromatography.[b] The cis-UCA fractions were evaporated to dryness and dried in a vacuum desiccator over NaOH. The purity of cis-UCA (as determined via HPLC) was 99.9%. The identity of cis-UCA was also characterized via infrared, hydrogen 1 (^1H), and carbon 13 (^{13}C) nuclear magnetic resonance spectrometric methods, UV-visible spectrophotometry, and melting point analysis. The endotoxin concentration was < 24.9 U/g as determined by use of the *Limulus* amebocyte lysate gel-clot method.

Study design—The study had a 3 × 3 Latin square crossover design. The purpose was to evaluate the effects of saline (0.9% NaCl) solution[c] (control treatment) and 2 concentrations of cis-UCA (12.5 or 25mM) on variables indicative of tissue inflammation and injury in *E coli*–inoculated mammary glands of lactating dairy cows. Only glands from which milk samples were free of detectable bacterial growth and contained milk SCCs < 200,000 cells/mL were used in the study. Prior to the start of the first experiment, the 12 selected cows were randomly assigned to either the control group or one of the cis-UCA treatment groups (4 cows/group). The right rear mammary gland of each of the 12 animals was experimentally inoculated with *E coli* at 0 hours. At 6, 12, 18, 24, 30, and 36 hours after inoculation, the inoculated mammary gland in each of the 4 cows in each group was infused with 40 mL of saline solution, 12.5mM cis-UCA, or 25mM cis-UCA. Following completion of the first experiment, all cows were treated with intramammary infusions of hetacillin potassium and allowed to recover for 7 weeks.

After the recovery period, the second experiment was initiated. The left front mammary gland of each cow was experimentally inoculated with *E coli*, and the assigned treatment groups were rotated. Thus, in the cows that received saline solution during the first experiment, the mammary glands inoculated in the second experiment were treated with 12.5mM cis-UCA; in the cows that received 12.5mM cis-UCA during the first experiment, the mammary glands inoculated in the second experiment were treated with 25mM cis-UCA; and in the cows that received 25mM cis-UCA during the first experiment, the mammary glands inoculated in the second experiment were treated with saline solution. All other aspects of the second experiment were the same as those of the first experiment. Following completion of the second experiment, all cows were treated with intramammary infusions of hetacillin potassium and allowed to recover for 7 weeks.

After the recovery period, the third experiment was initiated. The right front mammary gland of each cow was experimentally inoculated with *E coli*, and the assigned treatment groups were rotated. Thus, in the cows that received saline solution during the second experiment, the mammary glands inoculated in the third experiment were treated with 12.5mM cis-UCA; in the cows that received 12.5mM cis-UCA during the second experiment, the mammary glands inoculated in the third experiment were treated with 25mM cis-UCA; and in the cows that received 25mM cis-UCA in the second experiment, the mammary glands inoculated in the third experiment were treated with saline solution.

All other aspects of the third experiment were the same as those of the first 2 experiments.

Experimentally induced intramammary infection—*Escherichia coli* strain P4, which was originally isolated from a cow with naturally occurring mastitis,[42] was used to induce intramammary infection in the study cows in each of the experiments. The inoculum of *E coli* used was prepared as previously described.[17] Briefly, 10 mL of brain heart infusion broth[d] was inoculated with *E coli* strain P4 and incubated for 6 hours at 37°C while shaken at 225 rpm. Two milliliters of the culture was then transferred to an aerating flask containing 198 mL of tryptic soy broth[d] and incubated overnight (approx 16 hours) at 37°C while shaken at 225 rpm. The flask was placed in an ice water bath and mixed by swirling. An aliquot of the culture fluid from the flask was serially diluted, and the resulting dilutions were plated on blood agar plates.[d] The plates were incubated overnight and bacterial colonies were subsequently enumerated. The aerating flask containing the stock culture was maintained at 4°C overnight. After determining the concentration of the stock culture on the basis of the plate counts, the stock culture was diluted in sterile PBS solution. To induce experimental infection, 3 mL of the diluted culture fluid was infused into 1 mammary gland of each cow in each experiment. Results of plating of the final inoculums on blood agar plates confirmed that cows received 777, 195, and 233 CFUs of *E coli* in the first, second, and third experiments, respectively. Subsequently, successful establishment of infection was determined on the basis of culture of *E coli* from milk samples that were aseptically collected from the experimentally inoculated mammary glands. Only mammary glands in which an infection was successfully established were included in the study.

Analysis of cis-UCA in milk samples—Immediately prior to (0 hours) and at 12, 18, 24, 30, 36, 48, and 72 hours after inoculation with *E coli*, milk samples were collected from the experimental mammary glands. At sample collection time points that coincided with experimental treatment, samples were collected before saline solution or cis-UCA was infused. Prior to sample collection, each gland was sprayed with an iodine-based disinfectant, forestripped, dried with a paper towel, and scrubbed by use of sterilized gauze pads that were saturated with 70% ethanol. Following this procedure, milk samples were collected into sterile tubes and frozen. For analysis of milk concentrations of cis-UCA, the frozen samples were allowed to thaw at 4°C overnight and centrifuged at 6,500 × *g* for 15 minutes at 4°C. The fat layer was removed, and the skimmed milk was subjected to protein precipitation via addition of an equal volume of a reagent containing 3 parts of methanol-aqua mixture (1:5 [vol/vol]) and 2 parts of 40% trichloroacetic acid. The samples were incubated at 0°C for 10 minutes, and centrifugation was performed at 4°C. The supernatants were analyzed in duplicate by use of an HPLC system[e] with an NH_2 column (250 × 4.6 mm; 5 μm)[f] and detection wavelength of 268 nm. The eluent was an acetonitrile-0.05M KH_2PO_4 mixture (50:50 [vol/vol]; flow rate, 1 mL/min).

Determination of intramammary *E coli* growth and milk SCCs—Immediately prior to (0 hours) and at 6, 12, 18, 24, 30, 36, 48, 72, 96, and 168 hours after inoculation, milk samples were aseptically collected from experimental mammary glands. At time points that coincided with the cows' routine morning and evening milkings, samples were collected immediately prior to milking. For aseptic sample collection, each gland was sprayed with an iodine-based disinfectant, forestripped, dried with a paper towel, and scrubbed by use of sterilized gauze pads that were saturated with 70% ethanol. Following cleaning and disinfection of the mammary glands, milk samples were collected into sterile tubes for analysis of bacterial growth, SCC, and biomarkers of inflammation and injury. For the determination of milk concentrations of *E coli*, aliquots of milk samples were aseptically collected from experimental mammary glands at various time points and serially diluted in sterile PBS solution; 100-μL volumes of each of the resulting dilutions were spread on 5 blood agar plates. Following a 24-hour incubation at 37°C, plates were examined for bacterial growth and colonies were enumerated. For the quantification of somatic cells, milk samples were heated to 60°C for 15 minutes and subsequently maintained at 40°C until somatic cells were counted by use of an automated cell counter.[g]

Whey preparation—For the preparation of whey, each milk sample was centrifuged at 44,000 × *g* at 4°C for 30 minutes and the fat layer was removed with a spatula. The skimmed milk was decanted into a clean tube and centrifuged again for 30 minutes, and the translucent supernatant was collected and stored at −70°C.

Determination of BSA and TNF-α concentrations and LDH and NAGase activities in whey fractions—The BSA and TNF-α concentrations in the whey fractions of collected milk samples were determined by use of ELISAs as previously described.[17,43] For determination of LDH activity, the whey fractions of collected milk samples were diluted 1:3 in PBS solution containing 1% BSA and assayed as previously described[32] by use of a commercially available kit[h] and a multimodal plate reader.[i] For determination of NAGase activity, the whey fractions of collected milk samples were assayed as previously described[44] with only slight modification. Briefly, whey samples were diluted 1:2 in deionized H_2O, and 10 μL of the resulting dilution was pipetted into a black, clear-bottom, 96-well plate. Forty microliters of substrate solution (2.25mM 4-methylumbelliferyl N-acetyl-beta-D-glucosaminide[j] dissolved in a 0.25M sodium citrate solution [pH, 4.6]) was then added to the wells, and the plate was incubated for 15 minutes in the dark at 25°C while being shaken at 150 rpm. Immediately following the incubation period, 150 μL of stop buffer (0.2M glycine [pH, 10.8]) was added to each well. The fluorescence within each well was then measured by use of a multimodal plate reader[i]; the excitation wavelength was 360 nm, and the emission wavelength was 460 nm. The NAGase activities of the samples were extrapolated from the fluorescence values of a standard solution (1:2 dilutions of 4-methylumbelliferone[j] in 0.2M glycine [pH, 10.8]) that was assayed in parallel. To determine the amount of activity (nmol/mL/min), the extrapolated values were multi-

plied by the dilution factor (ie, 2) and multiplied by a factor of 100 to calculate the activity in 1 mL of sample, and the resulting product was divided by 15 to calculate the activity per minute.

Statistical analysis—A repeated-measures ANOVA was performed by use of computer software[k] to test for significant treatment, time, or treatment-time effects and to model and remove any treatment carryover effects[45] for each dependent variable. When present, correlations among repeated measurements across time were modeled by use of compound symmetric or first-order autoregressive covariance structures, as appropriate, for each dependent variable analyzed. Because residual variances were heterogeneous at the 36- to 96-hour time points and between the control treatment and the 2 cis-UCA treatments, they were modeled as necessary for some dependent variables. Pairwise means comparisons among the 3 treatments were conducted at each time point. In the absence of a significant treatment-time effect, pairwise means comparisons among the 3 treatments were conducted for all time points combined during the treatment period (12 to 36 hours) and during the entire experimental period (0 to 168 hours) by use of a Sidak adjustment (least squares means option) to maintain an experiment-wise error rate of 0.05. A value of $P < 0.05$ was considered significant.

Results

To evaluate the effects of saline solution (control treatment) and 12.5 and 25mM solutions of cis-UCA on the inflammatory response to *E coli*–associated mastitis in cows, the 12 study cows each underwent 3 experiments; in each experiment, a different mammary gland was inoculated with *E coli* and 1 of the 3 treatments was applied. Thus, the experiment was designed so that each treatment was applied to each of 12 mammary glands on 6 occasions (ie, at 6, 12, 18, 24, 30, and 36 hours after intramammary inoculation with *E coli*). Prior to the start of each experiment, milk samples were aseptically collected from each mammary gland and evaluated for microbial growth and SCC. Only glands from which milk samples were free of detectable bacterial growth and contained milk SCCs < 200,000 cells/mL were used in the study. During the study, 4 mammary glands did not meet these criteria prior to the start of a given experiment, and in another 4 glands, an infection was not established following the intramammary infusion of an *E coli* inoculum. Also, 1 cow that was in the control group in the first experiment developed health complications after the completion of that experiment and subsequently aborted its calf; this cow was not used in the second and third experiments. Therefore, the total number of mammary glands evaluated in the study was 26 (not 36 as anticipated), of which 7, 10, and 9 glands were treated with saline solution, 12.5mM cis-UCA, and 25mM cis-UCA, respectively.

Intramammary concentrations of cis-UCA—Following treatment of *E coli*–inoculated mammary glands with 12.5 or 25mM cis-UCA, analysis of milk samples for cis-UCA concentration revealed a 2-phase temporal profile. In the first phase from 12 to 24 hours, the concentrations reached a peak level of approximately 0.22mM (0.03 mg/mL) and 0.72mM (0.1 mg/mL) after infusions of 12.5 and 25mM cis-UCA, respectively (**Figure 1**). In the second phase from 30 to 36 hours, approximately 2-fold higher concentrations of cis-UCA were detectable in milk from treated glands; a mean peak concentration of 1.9mM (0.26 mg/mL) was evident at 36 hours following repeated infusions of 25mM cis-UCA. The corresponding peak concentration in mammary glands that received infusions of 12.5mM cis-UCA was 0.68mM (0.09 mg/mL). Within 36 hours of the last treatment (ie, at 72 hours after *E coli* inoculation), the concentration of cis-UCA in milk was nondetectable in cows that received either cis-UCA treatment. Analysis of milk samples collected from all cows regardless of treatment immediately prior to *E coli* inoculation (0 hours), as well as analysis of milk samples collected at 24 hours after inoculation from all cows receiving the control treatment, revealed no measurable concentrations of cis-UCA.

Effect of treatments on bacterial growth in *E coli*–inoculated mammary glands—To evaluate the effect of cis-UCA on intramammary bacterial growth, milk samples were collected prior to intramammary infusion of *E coli* (0 hours) and at various subsequent time points (ie, after treatment had been initiated) for analysis. Regardless of treatment group, bacterial culture of milk samples from all inoculated mammary glands yielded *E coli* at the 12-hour time point (**Figure 2**). Throughout the study, *E coli* had been eliminated from all glands by the end of each experimental period with the exception of 2 mammary glands that were treated with 12.5 or 25mM cis-UCA and 1 gland that was treated with saline solution. In mammary glands that were infected with *E coli*, significant increases in the milk bacterial concentrations, compared with findings at 0 hours, were detected in all 3 treatment groups at 6 to 72 hours after inoculation of the mammary glands (**Figure 3**). During the treatment period (12 to 36 hours), as well as

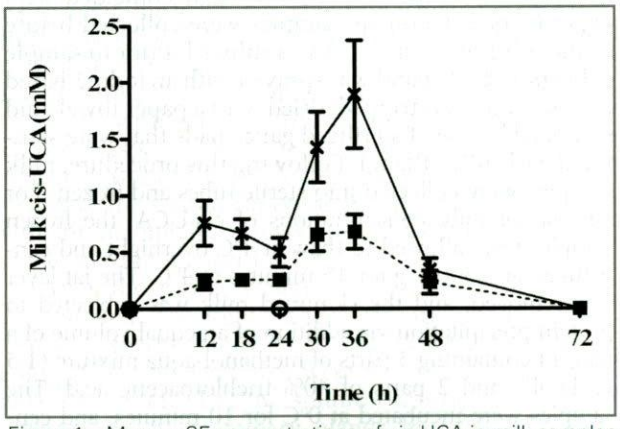

Figure 1—Mean ± SE concentrations of cis-UCA in milk samples collected from bovine mammary glands (1 gland/cow) infected with *Escherichia coli* that were repeatedly infused with saline (0.9% NaCl) solution (circles; n = 7), 12.5mM cis-UCA (squares; 10), or 25mM cis-UCA (crosses; 9). Mammary glands were each inoculated with *E coli* (mean inoculum, 400 CFUs) at 0 hours and subsequently infused with 40 mL of saline solution or cis-UCA at 6, 12, 18, 24, 30, and 36 hours. Milk samples for cis-UCA analysis were collected immediately prior to and at 12, 18, 24, 30, 36, 48, and 72 hours after gland inoculation.

throughout the entire experimental period (0 to 168 hours), there were no significant treatment effects of cis-UCA on bacterial concentrations in treated glands.

Effect of treatments on inflammatory responses to intramammary *E coli* infection—In the study cows, rectal temperatures were measured and recorded prior to inoculation of mammary glands with *E coli* (0 hours) and at various subsequent time points (ie, after treatment had been initiated). At 18 hours after inoculation, mean rectal temperature in cows in each of the 3 treatment groups was significantly increased, compared with the preinoculation (0-hour) measurement (**Figure 4**). However, among the 3 treatment groups, mean rectal temperatures at 18 hours after inoculation were comparable. At 24 hours after inoculation, rectal temperatures in all 3 treatment groups had returned to 0-hour values. During the treatment period (12 to 36 hours), as well as throughout the entire experimental period (0 to 168 hours), there were no significant treatment effects of cis-UCA on the febrile response induced by intramammary infection.

Milk samples were also analyzed for SCC and BSA and TNF-α concentrations during the study. Compared with findings before inoculation with *E coli*, milk SCCs were increased in all experimental mammary glands at 18 to 168 hours, regardless of which treatment was administered to the cows (Figure 4). Maximal increases in SCC in glands receiving either of the cis-UCA treatments did not significantly differ from the maximal increase detected in glands receiving the saline solution treatment. Similar to the changes in SCC, milk concentrations of BSA and TNF-α were increased in all experimental mammary glands at 18 hours after inoculation with *E coli*, regardless of which treatment was administered to the cows. Maximal increases in BSA or TNF-α concentration in glands receiving either of the cis-UCA treatments did not significantly differ from the maximal increase detected in glands receiving the saline solution treatment. However, in glands treated with 25mM cis-UCA, peak concentrations of both BSA and TNF-α were consistently lower (by approx 25%) than the corresponding peak concentration in the saline solution–treated glands.

Prior to the initiation of the study to investigate the effects of cis-UCA on inflammation and injury associated with *E coli* intramammary infection, a pilot study was conducted to investigate the clinical effects of intramammary infusion of 25mM cis-UCA in 2 healthy mammary glands of an individual cow. Compared with findings in 2 mammary glands of the same cow that were infused with saline solution, the glands infused with cis-UCA did not develop local clinical signs of inflammation or alterations in milk SCC (data not shown). Furthermore, no systemic clinical signs developed in response to infusion of cis-UCA alone, as evidenced by the lack of induction of fever and the absence of changes in milk production in this cow.

Effects of cis-UCA on tissue injury induced by intramammary *E coli* infection—To evaluate the ability of cis-UCA to protect against the tissue injury associated with mastitis, activities of 2 markers of epithelial injury (NAGase and LDH) were assayed in milk samples collected throughout the experimental period. Compared with the preinoculation findings (0 hours), significant increases in milk LDH activity were detected in glands in all 3 treatment groups at 18 to 96 hours after *E coli* inoculation (**Figure 5**). A significant effect of cis-UCA treatment on overall milk LDH activity was detected during the treatment period (12 to 36 hours; $P = 0.008$), as well as throughout the entire experimental period (0 to 168 hours; $P = 0.002$). During the treatment period, overall LDH activity in glands treated with saline solution was significantly higher than values in glands that were treated with 12.5mM ($P = 0.014$) or 25mM ($P = 0.007$) cis-UCA. Similarly, analysis of data

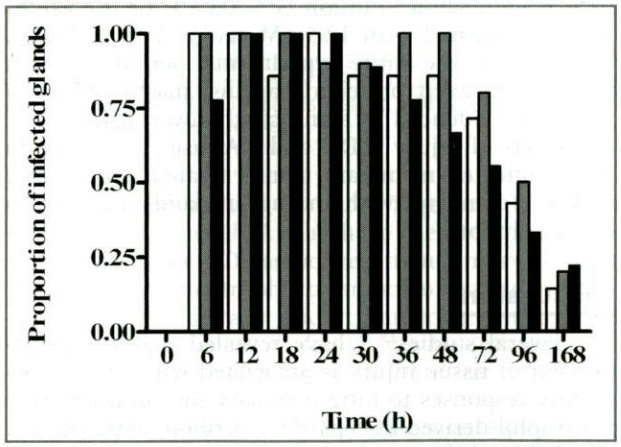

Figure 2—Proportion of infected mammary glands following inoculation of bovine mammary glands with *E coli* (1 gland/cow) and their subsequent treatment with saline solution (white bars; n = 7), 12.5mM cis-UCA (gray bars; 10), or 25mM cis-UCA (black bars; 9). Milk samples were aseptically collected for bacterial culture from experimental glands immediately before (0 hours) and at intervals (from 6 to 168 hours) after inoculation with *E coli* (mean inoculum, 400 CFUs); after sample collection at 6 through 36 hours, mammary glands were infused with 40 mL of saline solution or cis-UCA solution.

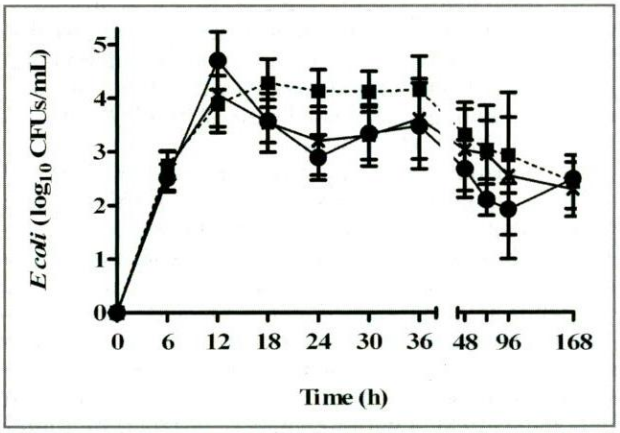

Figure 3—Growth of *E coli* (mean ± SE logarithm base 10 [log_{10}] CFUs/mL) in bovine mammary glands (1 gland/cow) in which infection was established following inoculation with *E coli*. Milk samples were aseptically collected for bacterial culture from experimental glands immediately before (0 hours) and at intervals (from 6 to 168 hours) after inoculation with *E coli* (mean inoculum, 400 CFUs); after sample collection at 6 through 36 hours, mammary glands were infused with 40 mL of saline solution, 12.5mM cis-UCA, or 25mM cis-UCA. Bacterial concentrations during the overall treatment period (from 12 to 36 hours), as well as during the overall experimental period (from 0 to 168 hours), did not significantly differ among treatments. *See* Figure 1 for key.

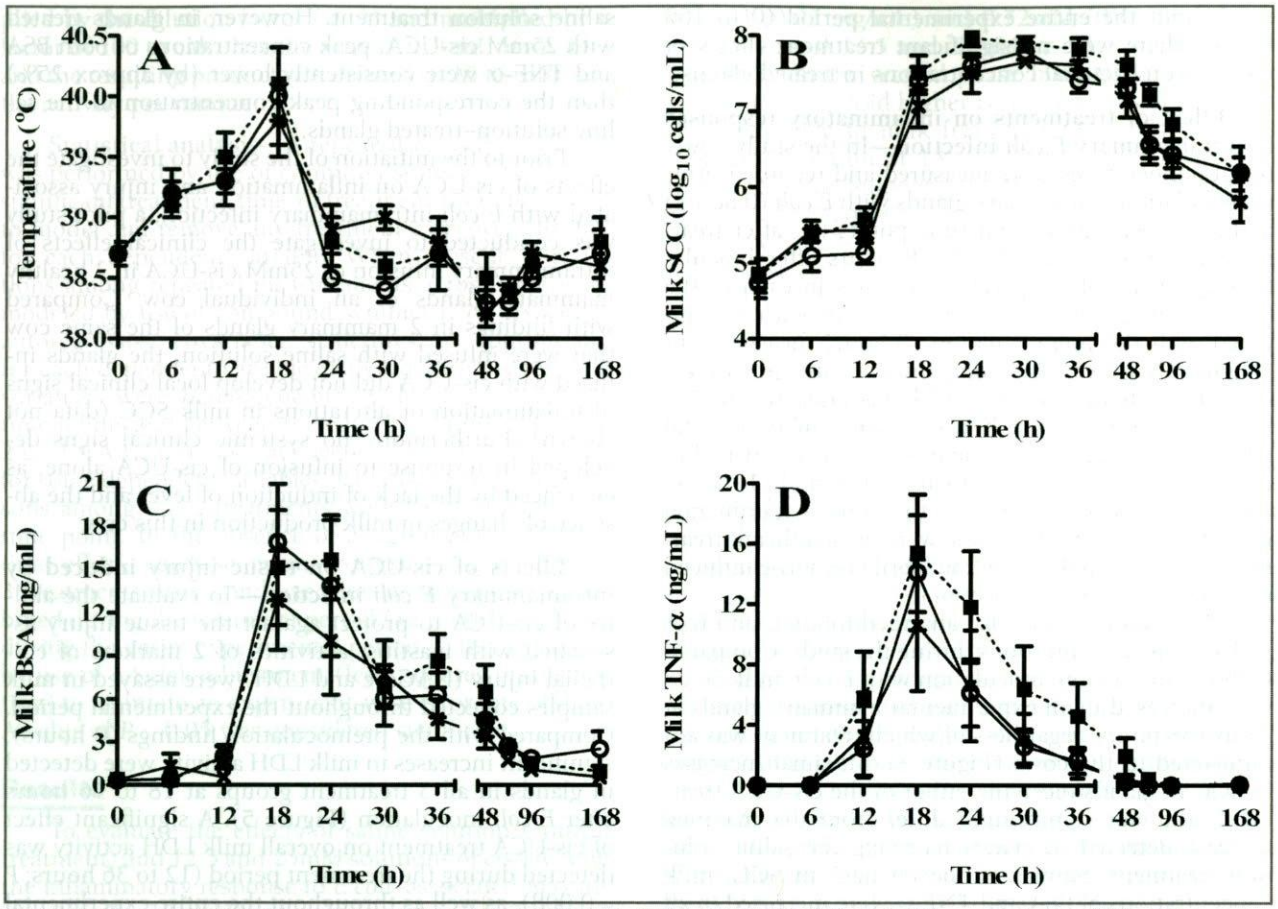

Figure 4—Mean ± SE rectal temperature (A) in cows and SCC (B), BSA concentration (C), and TNF-α concentration (D) in milk samples collected from their mammary glands (1 gland/cow) in which infection was established following intramammary inoculation with *E coli*. Immediately before (0 hours) and at intervals (from 6 to 168 hours) after inoculation with *E coli* (mean inoculum/gland, 400 CFUs), rectal temperature was measured and milk samples were collected from experimental glands. After sample collection at 6 through 36 hours, mammary glands were infused with 40 mL of saline solution, 12.5mM cis-UCA, or 25mM cis-UCA. Rectal temperature, SCC, BSA, and TNF-α responses during the overall treatment period (from 12 to 36 hours), as well as during the overall experimental period (from 0 to 168 hours), did not significantly differ among treatments. *See* Figures 1 and 3 for key.

collected throughout the experimental period revealed that overall LDH activity during this period was significantly greater in glands treated with saline solution, compared with glands treated with 12.5mM ($P = 0.009$) or 25mM ($P = 0.001$) cis-UCA.

Compared with the preinoculation findings (0 hours), significant increases in NAGase activity were detected in milk samples from glands in all 3 treatment groups at 24 to 96 hours after *E coli* inoculation (Figure 5). High variation in milk NAGase activity in the saline solution–treated glands precluded the detection of significant differences in overall NAGase activity in milk samples during the treatment period (12 to 36 hours) between glands treated with saline solution and glands treated with 12.5mM ($P = 0.996$) or 25mM ($P = 0.226$) cis-UCA. However, overall NAGase activity during this period was significantly ($P = 0.022$) lower in milk samples from glands treated with 25mM cis-UCA, compared with the activity in milk samples from glands treated with 12.5mM cis-UCA. Analysis of data collected throughout the entire experimental period (0 to 168 hours) revealed that overall NAGase activities during this period in glands treated with 25mM cis-UCA were significantly lower than the values in glands treated with saline solution ($P = 0.045$) or the values in glands treated with 12.5mM cis-UCA ($P = 0.008$). Thus, during the entire experimental period, cis-UCA was able to exert protection against mastitis-induced injury, as evidenced by significantly lower activities of 2 markers of injury (LDH and NAGase) in milk from *E coli*–infected mammary glands treated with 25mM cis-UCA, compared with findings in glands treated with saline solution.

Discussion

Several studies[23–26] have revealed that the development of tissue injury is associated with the inflammatory responses to intramammary infection and that neutrophil-derived ROS partly contribute to this injury. It is believed that extracellular release of ROS induces tissue injury, whereas intracellularly generated ROS are critical for neutrophil clearance of bacteria.[20,22,46] In previous in vitro investigations[40,41] by our group, we determined that cis-UCA reduces neutrophil respiratory burst activity and, more specifically, that it impairs extracellular, but not intracellular, generation of ROS. Also, cis-UCA does not impair neutrophil phagocytotic

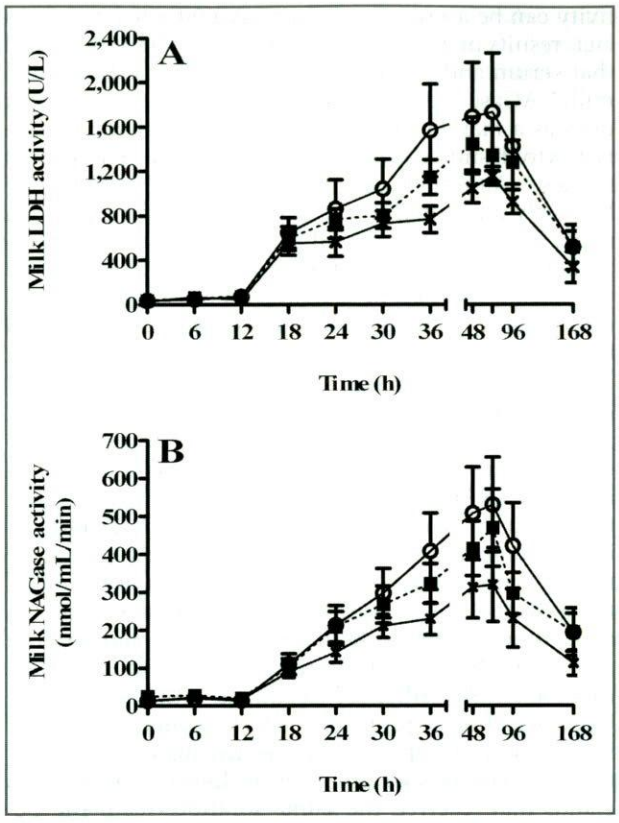

Figure 5—Mean ± SE activity of LDH (A) and NAGase (B) in milk samples collected from bovine mammary glands (1 gland/cow) infected with *E coli* that were repeatedly infused with saline solution, 12.5mM cis-UCA, or 25mM cis-UCA. Milk samples were aseptically collected from experimental glands immediately before (0 hours) and at intervals (from 6 to 168 hours) after inoculation with *E coli* (mean inoculum, 400 CFUs); after sample collection at 6 through 36 hours, mammary glands were infused with 40 mL of saline solution or cis-UCA solution. Activity of LDH during the overall treatment period (from 12 to 36 hours) was significantly higher in saline solution–treated mammary glands than in glands treated with 12.5mM cis-UCA ($P = 0.014$) or 25mM cis-UCA ($P = 0.007$). Activity of LDH during the overall experimental period (from 0 to 168 hours) was also significantly higher in saline solution–treated mammary glands than in glands treated with 12.5mM cis-UCA ($P = 0.009$) or 25mM cis-UCA ($P = 0.001$). Activity of NAGase during the overall experimental period (from 0 to 168 hours) was significantly higher in saline solution–treated mammary glands than in glands treated with 25mM cis-UCA ($P = 0.045$). *See* Figure 1 for key.

or bactericidal activity in vitro. Together, these data suggested that cis-UCA may have the therapeutic potential to reduce ROS-associated tissue injury without compromising neutrophil clearance of bacteria. Thus, the present study was undertaken to investigate the ability of cis-UCA to moderate in vivo inflammation and injury elicited in response to *E coli* intramammary infection in cows.

To investigate the in vivo effects of cis-UCA on intramammary infection in cows, *E coli* mastitis was experimentally induced in lactating dairy cows by use of an established method. The bacterial strain used to induce mastitis was originally isolated from a cow with naturally occurring mastitis, and the host innate immune response to intramammary infection with this strain has been extensively characterized.[9,17,47] In the present study, successful establishment of infection was determined on the basis of recovery of *E coli* from milk samples that were aseptically collected from the inoculated mammary glands. Only mammary glands in which an infection was successfully established were included in the study. The concentrations of cis-UCA used to evaluate its activity in vivo were derived from in vitro data; those data indicated that cis-UCA is able to reduce neutrophil-derived extracellular ROS production at concentrations as low as 0.01mM and that approximately 50% reduction is achieved with 0.85mM cis-UCA.[41] If one assumes an approximate residual volume of 0.25 L of milk/mammary gland, infusion of 40 mL of 12.5 or 25mM cis-UCA immediately after milking would be expected to result in intramammary milk concentrations of 1.7 or 3.5mM, respectively. On the basis of the recorded milk weights (data not shown) and a milking frequency of once every 12 hours, the approximate milk volume within the mammary glands of the cows in the present study immediately before milking (ie, when the mammary glands were full) was 3.3 L. Therefore, intramammary infusion of 12.5 and 25mM cis-UCA at the midpoint of the interval between consecutive milkings would be expected to yield milk concentrations of 0.3 and 0.6mM, respectively, in treated glands. The measured concentrations in samples collected 6 hours after the first infusion of 12.5 and 25mM cis-UCA were 0.24mM (0.033 mg/mL) and 0.76mM (0.105 mg/mL), respectively; these values indicated that the expected effective concentrations were attained. Moreover, in cows treated with 25mM cis-UCA, the concentration of cis-UCA in milk was similar to the concentration of cis-UCA (0.85mM) that inhibits 50% of neutrophil-derived extracellular ROS production. The frequency with which cis-UCA infusions were administered in the present study was determined from experimental findings, which indicated that cis-UCA is diluted to subtherapeutic concentrations in milk within 6 hours of infusion of a single dose into the mammary glands of dairy cows (authors' unpublished data). Thus, on the basis of the data from that kinetic experiment and findings of a previous investigation[17] that indicated that the acute inflammatory response to *E coli* intramammary infection develops within 36 hours, cis-UCA was administered to the cows of the present study every 6 hours throughout a 36-hour period following inoculation of mammary glands with *E coli*.

In the present study, the proportion of mammary glands that remained infected throughout the experimental period, as well as the concentration of *E coli* present in the milk of these glands, was comparable among treatment groups. The ability to clear an intramammary infection is mediated by both the absolute numbers of neutrophils recruited to the site of infection and the rapidity with which they are recruited.[48–51] During acute clinical mastitis, > 90% of milk somatic cells are neutrophils and the overwhelming increase in SCC can be attributed to neutrophil recruitment from the circulation.[52] Thus, the findings that cis-UCA did not have any overall effect on the magnitude of the milk SCC response and did not delay initial increases in SCC are consistent with the findings of equivalent milk bacterial concentrations and proportions of mammary glands that remained infected among treatment groups.

Together, these data suggest that cis-UCA does not impair host clearance of *E coli* and are consistent with an in vitro study[41] in which inhibition of neutrophil-mediated killing of bacteria by cis-UCA was not detected.

In addition to the role that neutrophils have in the innate immune response to intramammary infection, other inflammatory processes contribute to host clearance of pathogens. A diminished inflammatory response can lead to the establishment of chronic infection, and a more pronounced inflammatory response can enhance bacterial clearance.[17,53,54] For example, infusion of endotoxin into mammary glands infected with *Staphylococcus aureus* induces enhanced production of the proinflammatory cytokine TNF-α and increased vascular permeability.[53] The initial increases in these indicators of inflammation in response to endotoxin temporally correspond with 10-fold reductions in milk *S aureus* concentrations, compared with findings in infected mammary glands treated with saline solution.[53] Interestingly, the reductions in bacterial concentrations are detected at time points at which the SCCs in saline solution– and endotoxin-infused glands are equivalent. This indicates that, in addition to neutrophils, other aspects of the inflammatory response are critical for host clearance of intramammary infections. It has been postulated that the vascular influx of soluble mediators (eg, complement components) into mammary glands is critical for host innate defense mechanisms and that the lack of inflammation and accompanying increase in vascular permeability can impair bacterial clearance.[55] In the present study, cis-UCA did not impair production of the proinflammatory cytokine TNF-α; furthermore, cis-UCA did not adversely affect the influx of vascular-derived soluble mediators, as indicated by equivalent milk BSA concentrations among all glands, regardless of treatment. The lack of a significant inhibitory effect of cis-UCA on in vivo TNF-α production is consistent with the findings of previous in vitro studies[56,57] in which TNF-α production in activated keratinocytes and monocytes exposed to low concentrations of cis-UCA was measured. However, the findings of the present study are in contrast to the results of another in vitro study,[39] which indicated that cis-UCA indirectly inhibits TNF-α production in monocytes through a prostaglandin-dependent process. Overall, the data obtained from cows in the present study suggest that aspects of the proinflammatory response that may be beneficial to the host are preserved in the presence of cis-UCA and that cis-UCA does not negatively impact host clearance of bacteria from mammary glands.

In addition to proinflammatory markers, the effects of cis-UCA on mastitis-associated tissue injury were investigated in the study of this report. Two biomarkers, NAGase and LDH, were used to assess mammary tissue injury. In several studies,[29,31,32,58,59] activities of both markers in milk increased during mastitis and correlated with inflammation and infection. N-acetyl-beta-D-glucosaminidase is an intracellular lysosomal enzyme that is released from injured cells.[60] Although it has been reported that the increases in milk NAGase activity during mastitis are partly associated with high concentrations of neutrophils,[60] various studies[61–65] have established that most of the increase in this enzyme activity can be ascribed to mammary epithelial injury. In fact, results of 2 independent studies[61,63] have indicated that serum and leukocytes account for < 15% of total milk NAGase activity. Similar to NAGase, LDH has been used as a marker of cell injury. Lactate dehydrogenase is a cytoplasmic enzyme that is expressed in almost all tissues.[66] Although increases in milk LDH activity may be partly attributed to the influx of neutrophils, such increases can also be directly ascribed to mammary epithelial injury.[59,67,68] Therefore, NAGase and LDH activities have been used in several studies[29,32,59,69,70] of mastitis in cattle as in vivo markers of mammary epithelial injury and disease severity.

In the present study, cis-UCA had a protective effect against mastitis-induced tissue injury in dairy cows. Analysis of data collected during the entire experimental period revealed that activities of both biomarkers of injury (NAGase and LDH) were significantly lower in mammary glands that were treated with 25mM cis-UCA, compared with activities in glands that were treated with saline solution. On the basis of results of the milk cis-UCA analyses, the intramammary cis-UCA concentration should be maintained at a minimum of 1 to 2mM throughout the treatment period to achieve this effect. Nevertheless, treatment with 12.5mM cis-UCA also had a protective effect—concentrations of LDH in milk samples from infected mammary glands that received this treatment were lower than those in glands that received the saline solution treatment. Because cis-UCA treatment had no significant effect on either milk SCC or BSA concentration, the comparatively decreased milk NAGase and LDH activities in cis-UCA–treated mammary glands cannot be simply attributed to an influence of cis-UCA on either of these biomarkers of inflammation.

Results of 2 previous in vitro studies[40,41] have indicated that generation of ROS by neutrophils, which has been implicated in inflammation-associated tissue injury, is decreased by cis-UCA. The ability of cis-UCA to exert this effect without impairing the bactericidal activity of neutrophils has been attributed to the selective inhibition by cis-UCA of extracellular, but not intracellular, generation of ROS.[41] The mechanism by which cis-UCA selectively exerts this effect remains unknown; however, it has been postulated that cis-UCA may directly scavenge extracellular ROS, inhibit cell membrane–associated nicotinamide adenine dinucleotide phosphate oxidase generation of ROS, or block the escape of ROS from phagosomes as they form but have not completely sealed during phagocytosis. Regardless of the mechanism of the action, the present study revealed that the activities of 2 indicators of in vivo tissue injury in inflamed mammary glands are lower following treatment with cis-UCA than they are following treatment with saline solution, which is consistent with in vitro data that indicate that cis-UCA blocks the generation of tissue injury–inducing ROS.[40,41] The findings of the study of this report do not preclude another mechanism of action by which cis-UCA may provide protection against inflammation-associated tissue injury because it is known that cis-UCA has anti-inflammatory properties other than ROS inhibition.[39,71] Overall, the data obtained in the present study provide in vivo

evidence that cis-UCA may have therapeutic value in the treatment of mastitis in cows through limitation of inflammation-associated injury to mammary gland tissue.

a. Acros Organics, Geel, Belgium.
b. AG 1-X8 resin (200 to 400 mesh, acetate form), Bio-Rad Laboratories Inc, Hercules, Calif.
c. B. Braun Medical, Melsungen, Germany.
d. Becton-Dickinson Diagnostic Systems Inc, Sparks, Md.
e. Agilent 1100 HPLC system, Agilent Technologies Inc, Santa Clara, Calif.
f. Spherisorb S5 NH2 column, Waters Corp, Milford, Mass.
g. Bentley Somacount 150, Bentley Instruments Inc, Chaska, Minn.
h. CytoTox 96 non-radioactive cytotoxicity assay, Promega Corp, Madison, Wis.
i. Synergy HT multi-detection microplate reader, Biotec Instruments Inc, Winooski, Vt.
j. Sigma-Aldrich Co, St Louis, Mo.
k. SAS 9.1 PROC GLIMMIX, SAS, version 9.1.3, SAS Institute Inc, Cary, NC.

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